



PATENT
ATTORNEY DOCKET NO. 50195/023003

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	James M. Robl et al.	Confirmation No.:	4828
Serial No.:	10/705,519	Art Unit:	1632
Filed:	November 10, 2003	Examiner:	Deborah Crouch
Customer No.:	21559		
Title:	TRANSGENIC UNGULATES HAVING REDUCED PRION PROTEIN ACTIVITY AND USES THEREOF		

DECLARATION OF DR. YOSHIMI KUROIWA TRAVERSING GROUNDS OF
REJECTION OVER LACK OF ENABLEMENT

Under 37 C.F.R. § 1.132 and regarding the rejection of claims 1-6 and 25-38 for lack of enablement, I declare:

1. I am an inventor of the subject matter that is described and claimed in the above-captioned patent application. My curriculum vita is attached.
2. The specification provides ample guidance for one skilled in the art to produce hemizygous and homozygous prion protein (PrP) knockout cells and bovines (see, e.g., page 46, line 11 – page 47, line 31). This section teaches how a knockout vector used to inactivate the prion locus was constructed and further describes general methods for homologous recombination to produce knockout cells and calves.
3. Hemizygous and homozygous PrP KO bovines cells were actually produced using the methods described in the specification (page 54, line 12 – page 61, line 17). As stated in the specification, hemizygous PrP^{1/-} cells were produced at a frequency of approximately 50%, and homozygous PrP^{-/-} cells were produced from hemizygous cells

at a frequency of approximately 5%. Figs. 44A and 44B illustrate the generation of the two prion protein knockout vectors used to produce homozygous knockout cells. The specification further provides the transfection procedures employed (page 56, lines 4-26) and methods of screening for targeted integrations (page 56, line 28 to page 57, line 25) to produce hemizygous cells. The specification further describes how to use such cells in cloning methods to produce stable cell lines (page 57, line 27 to page 58, line 26). The specification also provides methods of using the hemizygous cell lines to produce homozygous cells (page 58, line 28 to page 59, line 19). Experiments have confirmed that homozygous prion protein knockout cells were produced (page page 59, line 21 to page 61, line 17).

4. The methods described in the specification have also been used to produce surviving hemizygous and homozygous PrP knockout bovines. These bovines were produced from additional cell lines produced using the methods described in the specification. In this Exhibits reference in this section, the PrP gene is referred to as *PRNP*.

In these experiments, a male Holstein primary fetal fibroblast line, 6594, was transfected with the first and second KO vectors (pBPrP(H)KOneo and pBPrP(H)KOpuro vectors) to sequentially disrupt the two alleles of the PrP gene as described in Kuroiwa, Y. *et al.* Sequential targeting of the genes encoding immunoglobulin- μ and prion protein in cattle. *Nat Genet.* 36, 775-780 (2004) (a copy of which is attached). The methods described in Kuroiwa et al. are substantially the same as those described in the present application. PrP^{-/-} fetal cell lines were established at 40-60 days of gestation, and three of the PrP^{-/-} fetal cell lines (5211, 5232, and 4296) were recloned to produce calves. We obtained five (16 %), two (8.6 %), and five (26 %) calves from the cell lines 5211, 5232, and 4296, respectively, as shown in Table 1.

Table 1. Production of cloned calves from PrP^{-/-} fibroblast cell lines

Genotype	Cell line ID	No. of recipients implanted	No. of calves born (%) ^a	No. of calves survived (%) ^a
PRNP ^{-/-}	4296	19	5 (26)	5 (26)
PRNP ^{-/-}	5211	30	7 (23)	5 (16)
PRNP ^{-/-}	5232	21	2 (8.6)	2 (8.6)

^aPercentages were calculated by dividing the number of calves by that of recipients implanted

Exhibit 1a shows PrP^{-/-} cattle at 13 months of age. Exhibit 1b shows verification of PrP^{-/-} genotype by genomic PCR on ear biopsy fibroblasts (P, positive control; N, negative control). The PrP^{-/-} calf is positive to PCR with puroF14 x puroR14 and neoF7 x neoR7 primers, which are specific to the targeting events at both alleles of PrP gene. The calf is negative for wild-type alleles of PrP gene amplified with BPrPex3F x BPrPex3R primers. Collectively, all calves born proved to be PrP^{-/-}. Exhibit 1c shows disruption of mRNA expression by RT-PCR analysis in fibroblasts of PrP^{-/-} calves. To detect PrP mRNA, PrPmF3 x PrPmR3 primers were used. These data confirmed the disruption of PrP-specific mRNA expression in PrP^{-/-} calves. Exhibit 1d shows the absence of PrP^C protein by Western blot analysis in fibroblasts of a PrP^{-/-} calf. As a positive control, wild type (WT) calf was analyzed. As a negative control, protein extracts from mouse fibroblasts were used because the monoclonal antibody used is claimed to be specific to bovine PrP^C protein. Protein extracts from wild type calf show the presence of 33-35 kDa of bovine PrP^C protein in size, but no positive band from the PrP^{-/-} calf. As an internal positive control, its replica blot was probed with anti-β actin antibody. Exhibit 1e shows the absence of PrP^C protein in peripheral blood lymphocytes (PBLs) of a PrP^{-/-} calf. Exhibit 1f shows the absence of PrP^C protein in brainstem of PrP^{-/-} calves. We detected PrP-specific bands in the wild type calves, whereas no reaction was observed in PrP^{-/-} calves and negative control mouse fibroblasts. These data clearly demonstrate that the PrP gene is functionally inactivated in the PrP^{-/-} calves.

5. The bovines produced using the methods described in the specification have not had any health problems attributable to the cloning process.

PrP^{-/-} cattle were monitored for growth and general health status from birth up to 18 months of age. Mean birth weight was 46 kg and average daily gain was 0.91 kg/day to 10 months. Both values were in the normal range for Holstein bulls. Serum chemistry was evaluated at 6 months of age and compared with published reference ranges. All the values for PrP^{-/-} calves (n = 12) were well within the reference range (Supplementary Table 1), and obvious abnormalities were not observed.

Supplementary table 1. Serum chemistry values for PrP^{-/-} cattle

Parameter	Reference Range*	PrP ^{-/-} calves (mean ± S.E.M.)**
Albumin (g/dl)	2.2-4.4	3.24 ± 0.04
Aspartate (U/L)	39-123	82.08 ± 2.54
Calcium (mg/dl)	8.2-12.5	9.27 ± 0.14
Gamma glutamyl transpeptidase (U/L)	9-42	13.42 ± 1.37
Total protein (g/dl)	5.2-8.6	6.66 ± 0.20
Globulin (g/dl)	2.1-4.9	3.43 ± 0.18
Blood urea nitrogen (mg/dl)	5-27	7.75 ± 0.39
Creatine kinase (U/L)	43-1007	265.08 ± 30.97
Phosphate (mg/dl)	4.2-10.9	9.09 ± 1.19
Magnesium (mg/dl)	1.5-2.3	2.0 ± 0.03

*International Species Information System, *Bos taurus taurus*; Domestic cow/ox; Conventional U.S.A. units

**n = 12

General physical examinations, done at monthly intervals by licensed veterinarians, included the following parameters: body temperature, heart rate, heart sound, jugular vein distension, respiratory rate, respiratory sound, presence of cough, nasal discharge, eye abnormalities, appetite, general behavior (alert and active, sluggish, hyperactive), gait, posture, joints, hooves, feces (diarrhea, constipation), genitalia, and umbilical cord (dry, enlarged, inflamed, infected). All parameters were normal for all PrP^{-/-} cattle (n = 12), and all PrP^{-/-} cattle survived this observation period without any unusual health problems.

At 10 months of age, eight pairs of PrP^{-/-} and age, sex, and breed-matched wild type control cattle were further given an extensive clinical examination (consisting of 122 parameters). These examinations were done according to the diagnostic evaluation of

ruminants suspected of TSE (transmissible spongiform encephalopathy) as described in the European TSE guideline "Surveillance and diagnostic of TSEs in ruminants". The clinical evaluation included a general examination of all organic systems and a detailed examination of the nervous system. Examination of the nervous system focused on the following aspects: (i) evaluation of mental status, studied by observation of animal behaviour and reactions to stimulations (approaching, menace, sounds, and light); (ii) evaluation of sensory function in limbs and trunk that included the study of superficial sensitivity, medular reflexes, and conscious proprioception; (iii) evaluation of motor function in limbs and trunk by studying the muscular tone, motor irritability (presence of muscles fasciculation and tremor), and gait abnormalities; and (iv) evaluation of cranial nerves that was done by the observation of disorders in the corresponding innervated regions. All animals ($\text{PrP}^{-/-}$ and controls cattle) showed a good healthy status in the general clinical examination, and none showed significant alterations in the examination of the nervous system, except the following observation; a mild increased reaction to external stimulation (menace and sounds) was observed in 3/8 $\text{PrP}^{-/-}$ cattle compared to 1/8 control cattle. The response to external stimulation was recorded as positive when it was observed in three consecutive stimulations. This observation does not indicate an alteration of nervous system but only a mild nervous temperament, which was mainly observed in the knockout cattle.

Blood samples were taken for hematology from five pairs of $\text{PrP}^{-/-}$ and control cattle at 10 and 12 months of age. The means for various hematological parameters from the two samples were compared (Supplementary Table 2) between $\text{PrP}^{-/-}$ and control cattle and with published reference ranges. Overall, hematology analysis did not reveal obvious unusual characteristics in $\text{PrP}^{-/-}$ cattle at 10 or 12 months of age.

Supplementary table 2. Hematology values for PrP^{-/-} and control cattle

Blood Parameter	Reference Range*	PrP ^{-/-} calves (mean \pm S.E.M.)**	Control calves (mean \pm S.E.M.)**	P-value
White blood cell count ($10^3/\mu\text{l}$)	5.0-19.8	13.0 \pm 1.43	10.4 \pm 0.77	0.024
Red blood cell count ($10^3/\mu\text{l}$)	5.2-13.1	9.5 \pm 0.36	8.6 \pm 0.85	0.11
Hemoglobin (g/dl)	9.0-15.3	9.7 \pm 0.07	10.2 \pm 0.50	0.34
Hematocrit (%)	22.0-47.0	27.6 \pm 0.35	29.1 \pm 1.33	0.32
Mean corpuscular volume (fL)	30.2-70.2	29.1 \pm 0.24	34.1 \pm 0.49	0.0001
Mean corpuscular hemoglobin (pg/cell)	10.0-23.0	10.2 \pm 0.17	11.9 \pm 0.16	0.0001
Mean corpuscular hemoglobin content (g/dl)	28.0-38.1	35.2 \pm 0.39	35.0 \pm 0.19	0.28
Platelet count ($10^3/\mu\text{l}$)	193-582	330 \pm 22.7	331 \pm 24.4	0.99
Neutrophils ($10^3/\mu\text{l}$)	1.1-15.8	6.2 \pm 0.81	3.0 \pm 0.19	0.005
Band Neutrophils ($10^3/\mu\text{l}$)	0.08-0.58	0.02 \pm 0.01	0.005 \pm 0.001	0.126
Lymphocytes ($10^3/\mu\text{l}$)	2.3-10.1	5.9 \pm 0.34	6.7 \pm 0.29	0.12
Monocytes ($10^3/\mu\text{l}$)	0.09-1.26	0.4 \pm 0.07	0.5 \pm 0.09	0.851
Eosinophils ($10^3/\mu\text{l}$)	0.06-1.85	0.2 \pm 0.08	0.2 \pm 0.07	0.96
Basophils ($10^3/\mu\text{l}$)	0.04-0.08	0.02 \pm 0.01	0.04 \pm 0.04	0.582

*International Species Information System, *Bos taurus taurus*; Domestic cow/ox; Conventional U.S.A. units

**n = 5 for each group

To evaluate the impact of PrP^C deletion on calf development, we performed extensive histopathological analyses on two PrP^{-/-} and two wild type cattle at 14 months of age. Representative samples of skin, nasal turbinate, lung, liver, kidney, spleen, salivary gland, thyroid gland, tonsils (pharyngeal, palatine), thymus, reticulum, rumen, omasum, abomasum, intestines (ileum, colon), adrenal gland, pancreas, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), aorta, striated muscles (heart, tongue, masseter, diaphragm, triceps, psoas major, biceps femoris), testicle (from 2 animals), nictitating membrane, sciatic nerve, both trigeminal nerves and ganglia, pituitary gland, spinal cord (cervical, thoracic, lumbar), one eye with its optic nerve, and the whole brain were evaluated. At least 14 sections of various areas of the brain (including obex, pons, colliculi, cerebellum, hippocampus, thalamus, and cerebral cortex) of each animal were examined by light microscopy. Two sections of spinal cord at cervical, thoracic, and lumbar regions were also evaluated by light microscopy. Neither obvious abnormalities nor significant lesions were observed for any tissue in either of the two groups.

Cells of the immune system play an important role in the pathogenesis of prion diseases, and PrP^C expression is readily detected in immune cells. Therefore, we examined the effects of PrP^C deficiency on the immune system of PrP^{-/-} cattle at 12-13 months of age. We evaluated B cell and T cell populations in peripheral blood lymphocytes (PBLs) of PrP^{-/-} cattle by flow cytometry. Exhibits 2a-2d show flow cytometry in peripheral blood lymphocyte stained with (2a) anti-IgM and anti-CD21 antibodies, (2b) anti-IgM and anti-lambda light chain antibodies, (2c) anti-CD4 and anti-CD8 antibodies, and (2d) anti-CD3 and anti- $\gamma\delta$ T cell receptor antibodies. No differences were observed in any of these cell subsets between PrP^{-/-} and wild type cattle.

To address immune-competence, PrP^{-/-} cattle were immunized with ovalbumin (OVA), a T cell-dependent antigen. Exhibit 2e shows the antibody response to OVA protein antigen in PrP^{-/-} and WT cattle. Four PrP^{-/-} and four WT cattle were immunized with OVA twice at day 0 (V1) and day 21 (V2), and OVA-specific IgG antibody titers at 7 days post V2 were determined. Mean antibody titers of PrP^{-/-} group and WT group cattle and their SEM are shown. Statistical analysis using Student's t test showed no significant difference between PrP^{-/-} and WT cattle ($p = 0.9$)

It has been shown in *Prnp*^{-/-} mice that T cell proliferation and cytokine production induced by T cell mitogens is significantly affected, suggesting a role of PrP^C in T cell function. Therefore, PBLs were isolated from PrP^{-/-} cattle and stimulated with anti-CD3 antibody, concanavalin A (ConA), and phytohemagglutinin (PHA). Exhibit 2f shows *in vitro* mitogenic response of T cells in PrP^{-/-} and WT cattle. PBLs from four PrP^{-/-} and four WT cattle were cultured with medium only (Med) or stimulated with immobilized anti-CD3 monoclonal antibody (CD3), Con A (concanavalin A), or PHA (phytohemagglutinin) mitogens for 48 hours, and proliferation was measured by ³H thymidine incorporation. Mean of T cell response of PrP^{-/-} group and WT group cattle and their SEM are shown. Statistical analysis using Student's t test showed no significant difference between PrP^{-/-} and WT cattle ($p = 0.9$ for anti-CD3; $p = 0.4$ for Con A and $p = 0.7$ for PHA). In contrast to *Prnp*^{-/-} mice, no difference in T cell proliferation after T cell

mitogen stimulation was observed for PrP^{-/-} cattle when compared to similarly treated cells from wild type cattle.

Exhibit 2g shows intracellular cytokine analysis of IFN γ expression in PrP^{-/-} and WT control cattle by dual color flow cytometry. PBLs were stimulated by immobilized anti-CD3 monoclonal antibody for 72 hours, and intracellular IFN γ production was analyzed by surface CD3 and intracellular IFN γ (green) dual color immunofluorescent staining. Percentage of IFN γ + T cells are shown in the upper right quadrant. Exhibit 2h shows *in vitro* IFN γ production by PBLs in PrP^{-/-} and WT cattle. PBLs isolated from four PrP^{-/-} and four WT cattle were stimulated by (i) immobilized anti-CD3 monoclonal antibody or (ii) Con A for 72 hours, and secreted IFN γ in the culture supernatant was analyzed by calibrated bovine IFN γ ELISA. Mean of the IFN γ production (ng/ml) in PrP^{-/-} group and WT control cattle and their SEM are shown. Statistical analysis using Student's t test showed no significant difference between PrP^{-/-} and WT cattle (p = 0.5). These data indicate that the ablation of PrP^C expression does not appear to have deleterious effects on the immune systems in cattle.

The PrP^{-/-} bulls reached sexually maturity at a normal age, and semen was collected from two KO animals at 16 months of age to address fertility of PrP^{-/-} cattle. Sperm appeared morphologically normal and were capable of generating normal-appearing blastocysts by *in vitro* fertilization with oocytes derived from wild type cows. This result indicates that PrP^{-/-} bulls are reproductively normal and could be used for production of a population of PrP^{-/-} cattle for commercial application.

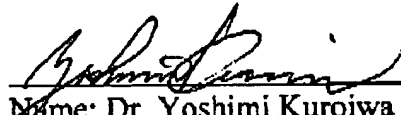
Collectively, these data indicate that disruption of the normal prion protein PrP^C expression in *Bos taurus* does not in any significant way adversely affect normal bovine development. PrP^{-/-} cattle remained healthy for at least 18 months after birth.

6. In my opinion, the specification provides ample guidance for one skilled in the art to produce hemizygous and homozygous PrP knockout cells and bovines. The methods described in the specification have been successfully employed to produce both cells and surviving calves. The frequency of success is also sufficient to allow one

skilled in the art to produce cells or calves reproducibly in 6 months for homozygously targeted cells or 15 months for homozygously targeted calves, after completion of vector construction.

7. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

11 Sep 2006
Date


Name: Dr. Yoshimi Kuroiwa